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(54) Title: CONTROL OF STEM CELL DIFFERENTIATION AND SELF RENEWAL BY MANIPULATION OF P38 OR NOTCH

(57) Abstract: The invention relates to methods for controlling precursor cell differentiation by treating a precursor cell with an agent that affects activity of mitogen-activated protein kinase (MAPK) p38 or signalling of Notch in the cell such that the cell undergoes differentiation or undergoes self renewal, and cell models for studying cell differentiation and self renewal.

CONTROL OF STEM CELL DIFFERENTIATION AND SELF RENEWAL BY MANIPULATION OF P38 OR NORTCH.

Technical Field

The present invention relates to assays for determining cell differentiation and self renewal and uses of the assays for developing or studying treatment modalities and agents.

Background Art

Stem cells

Stem cells refer to unique populations of cells in the embryo or adult, defined as clonogenic precursor cells capable of differentiation into at least one specialised cell type, as well as unlimited or prolonged self-renewal (Watt, F.M. & Hogan, B.L. Out of Eden: stem cells and their niches. *Science* 287, 1427-30 (2000)). The traditional view of stem cells is that they exist in tissues in which there is a constant turnover of post-mitotic differentiated cells, such as the blood system, skin, gut endothelium and skeletal muscle. In addition, they are mono- or oligo-potent, differentiating only into the tissue type in which they reside. While this view has changed radically over the last few years, the principles of stem cell function have been established in some of these systems. In the *Drosophila* gonad or PNS, stem cells can be recognised morphologically, and their spatial relationship to non-stem cell daughters is stereotypical. In other systems, there may be highly regulated mechanisms in which stem cells give rise to a daughter cell population in which the proportion of stem cells and more committed progenitors is determined on a probabilistic basis. Mechanistically, the two processes outlined above may be quite different, although both must certainly involve multiple feedback controls and intercellular dialogues. A key point is that population-based asymmetry allows for regulated responses to diverse physiological inputs. The molecular mechanisms that underlie control and regulation of stem cells remain largely unknown.

Whole bone marrow transplantation studies have revealed that hematopoietic precursor cells (HPC) are capable of differentiation into non-hematopoietic tissues, including endothelial cells, cardiac and skeletal muscle, liver oval cells and hepatocytes, and neuronal and glial cells of the central nervous system (CNS) (Goodell, M.A. Stem cells: is there a future in plastics? *Current Opinions in Cell Biology* 13, 662-665 (2001)). Subsequently, it was shown that neural, muscle and other stem cells could repopulate the hematopoietic system after lethal irradiation of recipients. Furthermore, the dual concept of plasticity and self-renewal was elegantly demonstrated by the long-term

repopulation of blood and multiple other organ lineages after transplantation of a single HPC in mice (Krause, D.S. et al. Multi-Organ, Multi-Lineage Engraftment by a Single Bone Marrow-Derived Stem Cell. *Cell* 105, 369-377 (2001)). These studies have lead to renewed investigations into stem cell mediated regenerative processes in organs such as the brain and heart, previously thought not to have significant regenerative capacity. In a rodent cardiac infarct model, various fractions of bone marrow enriched for HPCs and/or mesenchymal stem cells have now been shown to engraft the heart and participate in myocyte, endothelial cell and smooth muscle cell renewal, reducing apoptosis and scarring, and improving cardiac function (Orlic, D. et al. Bone marrow cells regenerate infarcted myocardium. *Nature* 410, 701-705 (2001)). It is now also clear that in the adult mammalian brain there are stem cells with the capacity to generate large numbers of neurons, in both normal life and during ischaemic injury (stroke) and trauma.

The source of adult stem cells remains controversial. As for germ cells, adult stem cells could in principle be held over from pluripotent stem cell populations in the embryo. However, many workers suggest that adult stem cells are derived from the bone marrow stem cell compartment. Indeed, dispersed muscle cells contain a stem cell population which expresses HPC markers and has 10-14 fold greater hematopoietic activity than whole bone marrow (Jackson, K.A., Mi, T. & Goodell, M.A. Hematopoietic potential of stem cells isolated from murine skeletal muscle. *PNAS* 96, 14482-14486 (1999)). Other workers suggest that adult stem cells are derived from endothelium, and have demonstrated that skeletal muscle precursor cells (MPCs) can be readily isolated from explants of dorsal aorta and from limbs of met-/- and Pax3-/- mice, into which somite-derived muscle precursors fail to migrate (De Angelis, L. et al. Skeletal Myogenic Progenitors Originating from Embryonic Dorsal Aorta Coexpress Endothelial and Myogenic Markers and Contribute to Postnatal Muscle Growth and Regeneration. *J. Cell. Biol.* 147, 869-877 (1999)). Consistent with this model is the fact that muscle satellite cells (see below) express endothelial markers. Currently, the prevailing view is that dispersed multipotent adult stem cells of unknown and perhaps multiple origins can transdifferentiate to a new tissue fate depending upon the environmental milieu in which they come to reside. There may be a genuine movement of stem cells throughout the body, akin to a stress response at the level of the whole organism rather than local tissue. Regardless of mechanism, the potential of the mammalian body for regeneration has now been convincingly demonstrated, and therapies based on transplantation of stem cells seem imminent.

Skeletal muscle stem cells

Skeletal muscle in vertebrates originates from precursor populations resident within somites, segmental mesodermal structures formed in the embryo. These cells give rise to the dermomyotome, then myotome, and form both appendicular and axial muscles. These muscles initially express high levels of MyoD and Myf-5, two members of the myogenic bHLH family of transcription factors, which also includes MRF-4 and myogenin. The other well-established precursor cell for skeletal muscle is the satellite cell (Seale, P., Asakura, A. & Rudnicki, M.A. The potential of muscle stem cells. *Dev Cell* 1, 333-342 (2001)). These cells arise late in development and account for the majority of post-natal and adult muscle growth, as well as the adaptive response of hypertrophy, and repair and regeneration after injury. Satellite cells have classically been described by their appearance as mononuclear cells located between the basal lamina and sarcolemma of myofibres. In the resting state, satellite cells are quiescent and most express distinct markers including c-met, M-cadherin, CD34, bcl2 and Pax-7. Satellite cells do not express high levels of the bHLH myogenic regulatory factors (MRFs) related to MyoD, although low-level expression of Myf5 in most (but not all) satellite cells has been detected using a Myf-5-lacZ reporter gene (Beauchamp, J. R. et al. Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. *J Cell Biol* 151, 1221-34 (2000)). Upon activation by stimuli such as injury, satellite cells rapidly upregulate expression of MyoD and/or Myf-5, and form muscle precursor cells (MPC). These undergo several rounds of replication, then differentiate after sequential expression of myogenin, $p21^{CIP1/WAF1}$ and myofilament genes, eventually fusing into multinucleate myotubes.

It has been shown that in addition to satellite cells and MPCs, skeletal muscle also contains a population of multipotential stem cells, which share properties with stem cells from a variety of other sources. As noted above, skeletal muscle contains cells that express hematopoietic stem cell markers and show blood lineage repopulation ability. As for HPCs, these cells also exclude Hoechst dye, giving rise to the so-called "side population" (SP) after FACS. McKinney-Freeman et al have argued that the cells with repopulating ability actually *are* HPCs and are clearly distinct from (although perhaps related to) cells with myogenic activity (McKinney-Freeman, S. L. et al. Muscle-derived hematopoietic stem cells are hematopoietic in origin. *PNAS* 99, 1341-1346 (2002)). How these populations relate to satellite cells is unknown, although they may exist in a hierarchical relationship, with some or all populations showing self-renewal (Gussoni, E. et al. Dystrophin expression in the mdx mouse restored by stem cell transplantation.

Nature 401, 390-4. (1999); Seale, P. et al. Pax7 is required for the specification of myogenic satellite cells. *Cell* 102, 777-86 (2000)). The various lines of evidence for heterogeneity in the stem cell populations within muscle, both in vitro and in vivo (Zammit, P.S. & Beauchamp, J. R. The skeletal muscle satellite cell: stem cell or son of stem cell? *Differentiation* 68, 193-204 (2001); Beauchamp, J. R., Morgan, J..E., Pagel, C. N. & Partridge, T. A. Dynamics of Myoblast Transplantation Reveal a Discrete Minority of Precursors with Stem-Cell-like Properties as the Myogenic Source. *J. Cell. Biol.* 144, 1113-1121 (1999); Baroffio, A. et al. Identification of self-renewing myoblasts in the progeny of single human muscle satellite cells. *Differentiation* 60, 47-57. (1996)), may relate to different states in this hierarchy. Pax-7 appears to be one important factor for specification of satellite cells, although not for that of the multipotential stem cell population.

Skeletal muscle stem cell self-renewal

The ratio of satellite cells as a percentage of total myonuclei drops from 32% at birth to <5% in rodents as a direct result of fusion to existing myocytes (Seale, P. & Rudnicki, M.A. A new look at the origin, function, and "stem-cell" status of muscle satellite cells. *Dev Biol* 218, 115-24. (2000)). In contrast, the number of quiescent satellite cells in adult muscle remains constant over several rounds of degeneration and regeneration, demonstrating the inherent capacity for self-renewal in this compartment. However, there is a significant progressive decline in satellite cell numbers during aging (Gibson, M.C. & Schultz, E. Age-related differences in absolute numbers of skeletal muscle satellite cells. *Muscle Nerve* 6, 574-580 (1983)), indicating that the self-renewal process is limited. This is also evident in the loss of satellite cell regenerative capacity in myopathies such as Duchenne muscular dystrophy, in which there are repeated bouts of injury and regeneration throughout life. Thus, self-renewal is an important although labile hallmark of regenerative stem cells. It is possible that many aging-related processes and disease states showing tissue atrophy may have as their causation a diminished capacity for stem cell renewal.

Muscle cell cultures

Primary cultures of activated satellite cells and myogenic cell lines are widely used as in vitro models of myogenesis. In such cultures, proliferation in a myoblastic phase occurs in high serum growth medium (GM), while differentiation can be induced in

a semi-synchronous manner in low serum differentiation medium (DM). A general feature of satellite cells induced to differentiate is the persistence of mononuclear cells that fail to differentiate, even after many days. This same property is seen in the established myogenic cell line C2C12, originally derived from mouse satellite cells (Yoshida, N., Yoshida, S., Koishi, K., Masuda, K. & Nabeshima, Y. Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates 'reserve cells'. *J Cell Sci* 111, 769-79. (1998)), suggesting that this line was derived from cells with self-renewal capacity. In C2C12 cultures, differentiation-resistant cells have been termed "reserve cells" and occupy the spaces between differentiated myofibres. While originating from MyoD-positive myoblasts, reserve cells rapidly lose expression of MyoD and remain largely quiescent (see Figure 1A). In the C2C12 line, such MyoD-negative cells represent about 50% of the culture. Enforced expression of MyoD eliminates reserve cell formation by triggering differentiation. Another important characteristic of reserve cells is their ability to re-express MyoD when returned to GM, and to generate both differentiated progeny and more reserve cells (see Figure 1B). Thus, reserve cells meet the current definition for stem cells: they are able to self-renew and yield in their progeny both cells committed to differentiate and cells similar to the mother cells. Various authors have found that reserve cells express markers also found on the majority of satellite cells, including CD34 and Myf-5. Satellite cells also heterogeneously express bcl-2, which appears to confer apoptosis-resistance to muscle stem cells (Dominov, J.A., Dunn, J.J. & Miller, J.B. Bcl-2 expression identifies an early stage of myogenesis and promotes clonal expansion of muscle cells. *J Cell Biol* 142, 537-44 (1998)). The mechanisms regulating reserve cell generation and down-regulation of MyoD expression are unknown, although the C2C12 system appears to represent a valid model for stem cell self-renewal. Kitzmann et al. found that Myf5 and MyoD accumulate in different phases of the cell cycle, with MyoD low in G0 and G1/M, suggesting that formation of reserve cells, which are MyoD-negative, may depend upon their position in, or place of exit from, the cell cycle (Kitzmann, M. et al. The muscle regulatory factors MyoD and myf-5 undergo distinct cell cycle-specific expression in muscle cells. *J Cell Biol* 142, 1447-59. (1998)).

The present inventors have determined the role of a number of cellular mechanisms in cell differentiation and self renewal which can be used to develop assays and determine the role of agents on the cellular differentiation/self renewal process.

Disclosure of Invention

In a first aspect, the present invention provides a method for controlling precursor cell differentiation comprising treating a precursor cell with an agent that affects activity of mitogen-activated protein kinase (MAPK) p38 or signalling of Notch in the cell such that the cell undergoes differentiation or undergoes self renewal.

Preferably, the precursor cell is a stem cell or a myogenic cell line. Preferably, the myogenic cell line is C2C12.

Preferably, the MAPK p38 is p38α or p38β.

In a preferred form, the agent is an inhibitor of MAPK p38 or an activator of MAPK p38.

Preferably the agent that affects activity of MAPK p38α or MAPK p38β is SB203580 (SB) (produced by Calbiochem) and the precursor cell is a stem cell or the myogenic cell line C2C12. It will be appreciated that other agents would also be suitable for the present invention. Other possible drug inhibitors of p38 MAPK include SKF 86002 and its derivatives and HEP689 and VX-745. (Lee, J.C., Kumar, S., Griswold, D.E., Underwood, D.C., Votta, B.J., and Adams, J.L. (2000) Inhibition of p38 MAP kinase as a therapeutic strategy. Immunopharmacology 47, 185-201). Other reagents activating or repressing the p38 pathway include: dominant-negative p38 (inhibitor), dominant-negative MKK6 (inhibits MKK6 which is the upstream activator of p38), constitutively active MKK6 (constitutively activates p38) (Raingeaud, J., Whitemarsh, A.J., Barrett, T., Derijard, B., and Davis, R.J. (1996) MKK3- and MKK6-regulated gene expressions mediated by the p38 mitogen-activated protein kinase signal transduction pathway. Molecular and Cellular Biology 16, 1247-1255).

Preferably the agent that affects Notch signalling in the cell is DFK-167 (Enzyme Systems Products) and the precursor cell is a stem cell or the myogenic cell line C2C12. It will be appreciated that other agents would also be suitable for the present invention. For example, Calbiochem produces the inhibitor MG132 and makes an inhibitor called gamma secretase inhibitor X. MDL28170 is cited in De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J.S., Shroeter, E.H., Schrijvers, V., Wolfe, M.S., Ray, W.J., Goate, A. and Kopan, R. (1999) A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. Nature 398, 518-522. Small molecule inhibitors (Compounds A-D, F and G) are reported in Seiffert, D., Bradley, J.D., Rominger, C.M., Yang, F., Meredith, J.E., Jn., Wang, Q., Roach, A.H., Thompson, L.A., Spitz, S.M., Higaki, J.N., Prakash, S.R., Combs, A.P., Copeland, R.A.,

Arneric, S.P., Hartig, P.R., Robertson, D.W., Cordell, B., Stern, A.M., Olson, R.E., and Zaczek, R. (2000) Presenilin-1 and -2 are Molecular Targets for Gamma-Secretase Inhibitors. Journal of Biological Chemistry 275, 34086-34091.

The present invention may lead to better therapies for stem cell-related diseases and loss of stem cell function during aging.

In a second aspect, the present invention is directed to a cell model for studying cell differentiation and self renewal comprising a cell modified to alter its sensitivity to an agent that affects activity of MAPK p38 or Notch signalling in the cell.

Preferably the cell is a precursor cell, more preferably a stem cell or C2C12 cell.

Preferably the agent that affects activity of MAPK p38α or p38β is SB203580 (SB) (produced by Calbiochem) and the precursor cell is a stem cell or the myogenic cell line C2C12. It will be appreciated that other agents would also be suitable for the present invention. Other possible drug inhibitors of p38 MAPK include SKF 86002 and its derivatives and HEP689 and VX-745. (Lee, J.C., Kumar, S., Griswold, D.E., Underwood, D.C., Votta, B.J., and Adams, J.L. (2000) Inhibition of p38 MAP kinase as a therapeutic strategy. Immunopharmacology 47, 185-201). Other reagents activating or repressing the p38 pathway include: dominant-negative p38 (inhibitor), dominant-negative MKK6 (inhibits MKK6 which is the upstream activator of p38), constitutively active MKK6 (constitutively activates p38) (Raingeaud, J., Whitemarsh, A.J., Barrett, T., Derijard, B., and Davis, R.J. (1996) MKK3- and MKK6-regulated gene expressions mediated by the p38 mitogen-activated protein kinase signal transduction pathway. Molecular and Cellular Biology 16, 1247-1255).

In another preferred form, the cell is modified to alter expression of MAPK p38α or p38β during pre-differentiation of the cell model during the time reserve cells are formed, or during post-differentiation of the cell model. The cell may be modified to over express, constitutively express, inducibly express, or substantially not express MAPK p38α or p38β.

As the present inventors have found that MAPK p38α or p38β plays a role in cell differentiation or self renewal, the cell model can be a useful tool in elucidating the cellular mechanism for cell differentiation or self renewal.

Preferably the cell is a modified precursor cell, more preferably a modified stem cell or a myogenic cell line, preferably C2C12.

Preferably the agent that affects Notch signalling in the cell is DFK-167 (Enzyme Systems Products) and the precursor cell is a stem cell or C2C12. It will be appreciated

that other agents would also be suitable for the present invention. For example, Calbiochem produces the inhibitor MG132 and makes an inhibitor called gamma secretase inhibitor X. MDL28170 is cited in De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J.S., Shroeter, E.H., Schrijvers, V., Wolfe, M.S., Ray, W.J., Goate, A. and Kopan, R. (1999) A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. Nature 398, 518-522. Small molecule inhibitors (Compounds A-D, F and G) are reported in Seiffert, D., Bradley, J.D., Rominger, C.M., Yang, F., Meredith, J.E., Jn., Wang, Q., Roach, A.H., Thompson, L.A., Spitz, S.M., Higaki, J.N., Prakash, S.R., Combs, A.P., Copeland, R.A., Arneric, S.P., Hartig, P.R., Robertson, D.W., Cordell, B., Stern, A.M., Olson, R.E., and Zaczek, R. (2000) Presenilin-1 and -2 are Molecular Targets for Gamma-Secretase Inhibitors. Journal of Biological Chemistry 275, 34086-34091.

In another preferred form, the cell is modified to alter Notch signalling during pre-differentiation of the cell model during the time reserve cells are formed, or during post-differentiation of the cell model. The cell may be modified to over express, constitutively express, inducibly express, or substantially not express components that play a role in Notch signalling of the cell.

As the present inventors have found that Notch signalling plays a role in cell differentiation or self renewal, the cell model can be a useful tool in elucidating the cellular mechanism for cell differentiation or self renewal.

In a third aspect, the present invention is directed to a method for assaying an agent for a capability of influencing cell differentiation or self renewal, the method comprising:

(a) providing a cell model according to the second aspect of the present invention;

(b) treating the cell model with an agent; and

(c) determining the affect of the agent on the differentiation or self renewal of the cell model.

The capacity for tissue regeneration via stem cells is an integral part of animal physiology. Furthermore, the clinical potential of stem cell therapies seems enormous, and the recent focus on adult stem cells avoids the sensitive ethical issues surrounding possible human therapies based on embryo-derived stem cells. Self-renewal is a key hallmark of all stem cells. This work is significant in that it seeks to define the molecular basis of stem cell self-renewal, seen as a dynamic equilibrium, in a muscle cell model. We believe that this information may be applicable to many stem cell systems. Stem cell

implantation to effect regeneration in disease, and manipulation of the self-renewal capacity of endogenous stem cells, may be equally valid therapeutic goals. Our studies are innovative in that we have discovered a key player in muscle stem cell self-renewal and have established a cell culture system that may be valuable in screening for new stem cell markers, and for new ways to manipulate the self-renewal equilibrium.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

In order that the present invention may be more clearly understood, preferred forms will be described with reference to the following drawings and examples.

Brief Description of the Drawings

Figure 1 is a schematic showing model for role of p38 during myogenisis (panel A) and model for reserve cells (panel B).

Figure 2 shows the results of SB treatment on a number of cell types and cell markers.

Figure 3 shows MLC p38 DR clone: (A) Differentiation at day 4 shows a complex mix of multinucleate myotubes (M - some outlined) and reserve cells (R) and; (B) differentiation at day 4 in the presence of SB 20 mM shows virtual "clearing" of reserve cells (*) with fusion into massive myosacs (F).

Figure 4 shows *Id(1-3)* mRNA expression is down-regulated during differentiation. Mean *Id1* mRNA expression (±SEM) from two independent microarray experiments in C2C12 myoblasts out to day 3 post-differentiation ±SB20 μM for (A) *Id1* (B) *Id2* and (C) *Id3*. All time-points are relative to a common reference composite sample and expression in each graph is relative to myoblast expression given by an arbitrary value of 1.0.

Figure 5 shows Western blot analyses of Id1 and Id2 protein expression. (**A**) Id1 and Id2 protein expression in C2C12 myoblasts and during differentiation ±SB 10 and 20 μM; α-tubulin present as loading control. (**B**) Id2 expression in myoblasts (MB) and at day 3 post-differentiation (D3)in MLC WT-6 and DR-1 p38 clones ±SB (20μM), α-tubulin present as loading control. Note that Id2 expression is present in myoblasts and at day 3 post-differentiation but not detectable in the presence of SB (D3). (**C**) Id2 expression in C2C12 myoblasts (MB), at day 3 post-differentiation (D3) either as whole cell lysate (Total) or divided into a mononuclear cell-enriched population or myotube-enriched population ±SB (20μM); α-tubulin present as loading control. Note that p-HSP27 is only detected in D3 cultures, and only in the myotube population.

Figure 6 shows Western blotting analyses of the expression of Id1 and Id2 and cell cycle-related proteins p21, cyclinD1 and PCNA, relative to tubulin control, in C2C12 cells after 3 days of differentiation in low serum in the presence and absence of SB and after fractionated into mononuclear cells (m) and myotubes (t).

Figure 7 shows inhibition of p38 with SB increases the number of thin and elongated myocytes and induces a loss of mononuclear cells in high density cultures of primary mouse myoblasts. Phase-contrast images of primary mouse myoblasts isolated then differentiated at high density in the absence (left panels) or presence of SB (20 μM) (right panels) for (**A** and **B**) one; (**C** and **D**) two and; (**E** and **F**) four days post-differentiation.

Mode(s) for Carrying Out the Invention

MAPK p38 and myogenesis

One part of the invention relates to the role of MAPK p38 in stem cell renewal. The MAP kinases comprise several families of intermediate signalling kinases which mediate a diverse range of cellular responses(Kyriakis, J.M. & Avruch, J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev* 81, 807-69. (2001)). p38 is one of the two stress-induced MAPKs, and has four isoforms (α,β, γ and δ), each the product of a different gene. They share 60–70% amino acid identity but vary in pattern of expression. The p38α and β isoforms are sensitive to the specific inhibitor SB203580 (SB), which interacts with Thr-106 in the hinge region of the ATP binding pocket (Wilson, K.P. et al. The structural basis for the specificity of pyridinylimidazole inhibitors of p38 MAP kinase. *Chem Biol* 4, 423-31. (1997)). p38 is classically activated by extracellular stresses such as inflammation,

UV irradiation and ischaemia/reperfusion, and by growth factors and cytokines such as FGF, IGF-1, IL1 and TNF-α. There are numerous downstream substrates identified for p38 including protein kinases such as MAPK-activated protein kinase 2 (MAPKAP-K2) and p38-activated kinase (PRAK), and transcription factors ATF-2 and MEF2A/C. Apart from mediating stress responses, p38 is involved in a number of unrelated processes (Nebreda, A.R. & Porras, A. p38 MAP kinases: beyond the stress response. *Trends Biochem Sci* 25, 257-260 (2000)), including *Drosophila* egg patterning and wing morphogenesis, cellular proliferation which may be both stimulatory or inhibitory depending on cell type, cellular survival and apoptosis, and cell differentiation of adipocytes, neurons, blood cells and myocytes. Several investigators have demonstrated the importance of p38 during muscle differentiation in vitro (Wu, Z. et al. p38 and extracellular signal-regulated kinases regulate the myogenic program at multiple steps. *Mol Cell Biol* 20, 3951-64. (2000)). p38 activity is low or absent in C2C12 myoblasts but increases during differentiation. Inhibition of p38 activity with SB blocks both myocyte fusion and differentiation. p38 activates the critical myogenic transcription factors MyoD and MEF2C, and is essential for activation of PI3 kinase pathway, also necessary for myogenic differentiation (Cuenda, A. & Cohen, P. Stress-activated protein kinase-2/p38 and a rapamycin-sensitive pathway are required for C2C12 myogenesis. *J Biol Chem* 274, 4341-6. (1999)). The knockout of p38α dies at mid-gestation due to erythropoietic and placental defects (Tamura, K. et al. Requirement for p38α in Erythropoietin Expression: A Role for Stress Kinases in Erythropoiesis. *Cell* 102, 221-231 (2000)).

Preliminary studies

The present inventors observed that over-expression of the novel muscle-specific cytoskeletal protein, Chisel, in C2C12 cells causes increased myocyte fusion during differentiation, but only in the presence of IGF-1 (Palmer, S. et al. The small muscle-specific protein Csl modifies cell shape and promotes myocyte fusion in an insulin-like growth factor 1-dependent manner. *J Cell Biol* 153, 985-98. (2001)). In searching for IGF-1 signalling intermediates that activate Chisel function, we noted a strong interaction between the p38 inhibitor SB and the pro-fusion phenotype. In the presence of SB, Chisel and IGF-1, virtually all cells in the culture fused into a giant "myosyncytium", despite the fact that SB is known to inhibit myocyte fusion and differentiation in normal cultures. In addition, we observed a dramatic "clearing" of the lawn of undifferentiated (reserve) cells in Chisel-expressing cultures in the presence of SB. This finding

suggested that the synergy between Chisel/IGF and SB was due to a combination of the pro-fusion effects of Chisel/IGF and a channelling of reserve cells into the myogenic (fusion-competent) program.

We undertook an examination of the role of p38 in normal myogenesis *in vitro*, and these studies are the basis of this invention. In the presence of SB (10-20 μM), there was a dose-dependent inhibition of myocyte fusion into multinucleate fibres. Resultant myocytes had one or only a few nuclei, even after 3 days, whereas controls without SB show extensive fusion and formation of myofibres, usually containing many tens of nuclei. The reserve cell clearing effect evident in SB-treated normal cultures, was complicated by the inhibition of fusion. Since reserve cells have been reported to be MyoD-negative, we assayed the number of MyoD-positive cells in cultures using immunofluorescence. We observed a down-regulation of MyoD in about 50% of cells after the onset of differentiation, and that MyoD-negative cells correspond to the undifferentiated reserve cells. However, there was a clear dose-dependent increase in MyoD-positive cells from day 1 of differentiation in the presence of SB (Figure 2). We also examined the downstream markers of myogenic commitment, myogenin and $p21^{CIP1/WAF1}$: these also increased, although with a delay of one day (Figure 2). Double immunostaining confirmed that only cells expressing MyoD were committed to differentiate, co-expressing myogenin, $p21^{CIP1/WAF1}$ or myosin heavy chain. Importantly, the greatest changes in the proportion of MyoD-positive cells in the presence of SB occurred at day 1 of differentiation, suggesting a role for p38 at the earliest stages of reserve cell creation.

We next cloned HA-tagged cDNAs encoding wild type (WT) oran SB-resistant (DR) variant of p38α into an expression plasmid driven by the myosin light chain (MLC) 1/3 promoter/enhancer, which is expressed only after the onset of myogenic differentiation. The DR mutant is relatively insensitive to the effects of SB due to three amino acid changes (Eyers, P.A., van den, I.P., Quinlan, R.A., Goedert, M. & Cohen, P. Use of a drug-resistant mutant of stress-activated protein kinase 2a/p38 to validate the in vivo specificity of SB 203580. *FEBS Lett* 451, 191-6. (1999)). We created and examined several C2C12 clonal lines with varying levels of expression of these genes. To summarise the results, high over-expression of WT p38α, or expression of the drug-resistant form, allowed us to bypass the inhibition of fusion conferred by SB. Notably, high levels of over-expression actually promoted fusion, and DR clones in the presence of SB (20 μM) differentiated into myofibres that were larger than normal. Critically, the reserve cell population was virtually eliminated in these cultures (Figure 3). At higher

doses of SB (40 μM), only clones expressing the highest levels of the p38α DR mutant were able to differentiate fully. Due to the complete lack of reserve cells, all cells in these cultures were differentiation and fusion-competent, and fused together into one massive myosyncytium. The data confirmed our previous findings using the Chisel-expressing cells that reserve cells are depleted in the presence of SB, and show that the initial sensitive period for inhibition of reserve cell formation by SB occurs prior to myogenic differentiation.

From the encouraging results in the cell model described above, it would be expected that similar effects would be observed in primary muscle satellite cell cultures that contain self-renewing cell precursors (see additional studies below).

Links between C2C12 reserve cells and *in vivo* muscle stem cells by examining expression of muscle stem cell markers

Several markers for muscle satellite cells have been described, including Myf-5, CD34, Pax7, c-met, m-cadherin and Bcl-2 (De Angelis, L. et al. Skeletal myogenic progenitors originating from embryonic dorsal aorta coexpress endothelial and myogenic markers and contribute to postnatal muscle growth and regeneration. *J Cell Biol* 147, 869-78. (1999)). We have obtained antibodies to all of these proteins from commercial sources or collaborators, and will analyse their status in reserve cells. We have obtained data for Pax7 and Myf5, and both appear to be expressed heterogeneously in the reserve cell population. This finding may relate to the observed heterogeneity in reserve cell self-renewal capacity observed in human primary muscle cultures, and to the fact that not all satellite cells express the above marker set. Early identification of the reserve cell will enable us to address the dynamics of their formation, and potentially to purify them by FACS for microarray and proteomics applications.

Notch and myogenesis

Notch is an evolutionarily conserved signalling system used by metazoans to control cell fate decisions during development (Artavanis-Tsakona, S., Rand, M., and Lake, R.J. (1999). Notch Signalling: Cell Fate Control and Signal Integration in Development. Science *284*, 770-776). The Notch protein is a single-pass transmembrane receptor activated by its membrane-tethered ligands Delta and Jagged. The transcription factor Suppressor of Hairless (Su(H); CBF1/RJBk in mammals) appears to be the main downstream effector of Notch signalling and transcription factors

of the Enhancer of split (E(spl)) family are their primary transcriptional targets. Post-translational processing appears to regulate the activities of the Notch receptor. α-secretase is a membrane-associated protein complex containing presenilin and nicastrin, which proteolytically cleaves Notch, liberating its intracellular domain and allowing it to passage to the nucleus. The activity of this complex is essential for activation of Notch signalling by its ligands.

The Notch signalling system controls an extraordinarily broad spectrum of cell fates and developmental processes and has been implicated in self-renewal of neural stem cells (Hitoshi, S., Alexson, T., Tropepe, V., Donviel, D., Elia, A.J., Nye, J.S., Conlon, R.A., Mak, T.W., Bernstein, A., and van der Kooy, D. (2002) Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells. Genes and Development *16*, 846-858). Notch has also been suggested to be important in skeletal myogenesis in that over-expression of a constitutively activate form of Notch inhibits myogenic differentiation in C2C12 myogenic cells in vitro (Kopan, R., Nye, J.S., and Weintraub, H. (1994). The intracellular domain of mouse Notch: a constitutively activated represser of myogenesis directed at the basic helix-loop-helix region of MyoD. Development *120*, 2385-96; Shawber, C., Nofziger, D., Hsieh, J.J., Lindsell, C., Bogler, O., Hayward, D., and Weinmaster, G. (1996). Notch signalling inhibits muscle cell differentiation through a CBF1- independent pathway. Development *122*, 3765-73).

We examined whether Notch signalling was involved in reserve cell formation. Using a Notch dependent luciferase reporter gene we observed that endogenous Notch activity was high in myoblasts, but fell progressively during their differentiation in low-serum medium. This suggests that Notch may play a role in maintaining the undifferentiated state in C2C12 cells. The α-secretase inhibitor DFK-167 (Enzyme Systems Products), has been previously used to inhibit Notch cleavage and thus activation of the pathway dependent on Notch ligands (Wolfe, M. S., Citron, M., Diehl, T.S., Xia, W., Donkor, I. O., and Selkoe, D. J. (1998). A substrate-based difluoro ketone selectively inhibits Alzheimer's gamma-secretase activity. J Med Chem *41*, 6-9; De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J.S., Schroeter, E.H., Schrijvers, V., Wolfe, M.S., Ray, W.J., Goate, A., and Kopan, R. (1999). A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. Nature *398*, 518-22). We employed this drug to inhibit Notch signalling in myoblasts and during differentiation. Morphological assessment shows that DFK-167, in a dose-dependent manner (30 and 50 μM), inhibits formation of reserve cell

in a way reminiscent of the clearing effects observed with SB203580. The loss of reserve cells was greatest when Notch inhibition was initiated in myoblasts compared to inhibition at the onset of differentiation. Unlike SB203580, which inhibits fusion of myocytes into multinucleate myotubes as well as reserve cell formation, DFK-167 appeared to induce precocious fusion, potentially through conversion of reserve cells to fusion-competent myocytes. When both SB203580 and DFK-167 were used together, we observed a greater degree of reserve cell clearing compared with use of either drug alone. These observations raise the possibilities that p38 and Notch act in parallel or the same pathway to determine reserve cell status.

It will be appreciated that DFK-167 will increase the number of MyoD+ cells during early phase of cell culture in which reserve cells are formed.

Microarray studies

cDNA microarray represents a powerful technology allowing mRNA expression to be examined on a very large scale. This technology has been made possible and manageable through advances in robotics, miniaturisation and bioinformatics. The resulting data generated represent a snapshot of the transcriptional state of the cell and current algorithms can cluster genes according to expression behaviour that may be involved in the same biochemical pathway.

Microarray technology has been applied to various aspects of biology and there have been several reports examining different aspects of myogenesis through microarray. These studies have examined the changes in gene expression during the different phases of myogenesis including proliferation of myoblasts and in differentiating and differentiated myocytes. Two of these studies utilised the C2C12 cell line as the model for myogenesis whilst the other one examined the effects of enforced MyoD expression into the mouse fibroblast cell line, C3H10T1/2.

Having discovered a fundamental and novel function for p38 in the C2C12 myogenic cell line, we embarked upon a microarray analysis of this model system. Our aim was to prove the principle that we could use the C2C12 system as modified by the inhibitory drugs under study to identify additional known and novel genes which may play a role in the specification and maintenance of the reserve cell population.

We compared the gene expression profile of C2C12 cells during normal proliferation and differentiation under conditions of SB (20 μM) treatment. From two preliminary experiments we identified a candidate family of genes, the *Id* (inhibitors of

DNA binding/differentiation) family, which is known to be important in the control of cellular proliferation and differentiation, and may be important in the regulation of the reserve cell. We then proceeded to quantitate protein expression of two of these genes, Id1 and Id2, and concluded that these genes may be important for reserve cell formation and/or maintenance.

Results

Methodology

We examined the transcriptional response of C2C12 cells during differentiation in the absence or presence of SB (20 μM). Since our previous data suggested that p38 activity may be required at both early and late phases of differentiation, we profiled mRNA expression at 0, 6, 10, 24, 48 and 72 hours post-differentiation. RNA from each time-point was labelled with Cy5 then co-hybridised with a Cy3-labelled reference pool of C2C12 mRNA that comprised approximately equal proportions from 0, 24, 48 and 72 hours post-differentiation. The labelled RNA was then co-hybridised to the NIA/NIH 15K mouse clone set. For each array spot, background-subtracted median spot intensities were calculated for each Cy-dye and the Cy5-Cy3 ratios within each array were adjusted to a median of 1.0 using intensity-dependent normalisation (Lowess). The level of expression of each mRNA was then defined relative to time 0 (myoblasts) which was designated as 1.0 for each gene. Two independent experiments were performed.

Genes were defined as present in the reference sample if the background-subtracted median pixel intensity for Cy3 was at least 50 in 9 or more out of the 11 samples in each experiment. This criteria was met by 5705 genes (37%) reflecting the expression bias of the array for which ESTs (expressed sequence tags) were derived from pre- and peri-implantation embryos, E12.5 female gonad/mesonephros, and newborn ovary rather than from skeletal muscle. The majority of these genes were not up-or down-regulated by more that 2.5-fold at any time with SB (78.8% of genes in experiment 1 and 87.3% in experiment 2).

We have found several changes in gene expression which could represent mechanisms of reserve cell regulation. Our data on the expression of *Id* genes, and the response to inhibition of p38 by SB are provided.

Id Family

The Id family of proteins (Ids) are important positive regulators of cellular proliferation and inhibitors of differentiation in various tissues. Ids contain a HLH domain which can heterodimerise with and sequester E-proteins from other bHLH transcription factors to negatively regulate differentiation, including MRFs in skeletal muscle. In addition, Id proteins lack the basic domain necessary for DNA binding. Furthermore, Id2, one of four Id isoforms in mammals, has been shown to exert an additional unique effect on cellular proliferation as it can bind pRb and induce release from E2F proteins. Id4 may also be able to target pRb but not Id1 or Id3. *Id* genes are widely expressed in various undifferentiated and proliferating cells during development but are down-regulated in differentiating cells.

Id genes have been previously shown to play important roles in myogenesis. *Id3* is co-expressed with *myogenin* in somites and developing muscles during mouse embryogenesis. In myogenic cultures, mRNAs for *Ids1-3* are expressed in proliferating myoblasts and down-regulated during differentiation. In addition, over-expression of *Id1*, *Id2* and *Id3* in myoblasts in vitro negatively regulates myogenic differentiation. Recently, *Id3* has been demonstrated to be under direct transcriptional regulation of MyoD in undifferentiated myoblasts. Our 15K NIA/NIH array chips contained mRNA of *Id1*, *Id2* and *Id3*; *Id4* was not represented although this isoform appears not to be expressed in skeletal muscle.

Microarray

The data are expressed as the mean fold change of mRNA expression from baseline (myoblasts) to time post-differentiation (6, 10, 24, 48 and 72 hours). The results are the mean (± SEM) from the two independent experiments. Consistent with previous data, we found that *Id1*, *Id2* and *Id3* expression was highest in proliferating C2C12 myoblasts and was rapidly down-regulated from the onset of differentiation, indicating that expression of these genes were sensitive to the effects of serum withdrawal (Figure 4). Inhibition of p38 with SB further suppressed expression of *Id1* and *Id2* but not *Id3*, with the greatest difference in expression seen for *Id2*.

Id1 and Id2 protein expression

To confirm the findings from our microarray data, we performed Western blot analyses of Id protein expression. Since our microarray results demonstrated that *Id1*

and *Id2* expression, but not that of *Id3*, was differentially regulated with SB, we examined the expression of Id1 and Id2 proteins in C2C12 cells ± SB during one to three days post-differentiation.

Id1 protein expression closely paralleled the mRNA expression results (Figure 5A). Expression was highest in myoblasts and was rapidly down-regulated by day 1 post-differentiation, and remained expressed at similar levels thereafter. SB induced a further reduction in expression in a dose-dependent manner at all time points post-differentiation.

Id2 protein expression was also very similar to the results from the microarray experiments. Id2 expression was highest in myoblasts and was down-regulated by day 1 post-differentiation (Figure 5A). Furthermore, inhibition of p38 with SB resulted in a greater reduction in Id2 expression with each day of differentiation. After three days of differentiation, there was virtually no detectable Id2 expression in the SB-treated cultures. These data raised the possibility that Id2 expression may be restricted to reserve cells since SB induces a diminishment of this cell population. Alternatively, Id2 expression may be dependent on the formation of multinucleate myotubes which are absent in the presence of SB.

To examine the hypothesis that Id2 may be restricted to the reserve cell population, we separated the myotube and mononuclear cell compartments from a well-differentiated C2C12 culture, and examined each population for Id2 expression. We lightly trypsinised well-differentiated C2C12 cultures in which myotubes are more sensitive to typsinisation and therefore can be differentially separated. It is noteworthy that with this technique of differential trypsinisation, the myotube-enriched population will contain some mononuclear cells and vice versa. In addition, whilst most of the mononuclear cell population will be reserve cells, some will be MyoD-positive.

We found that Id2 expression was restricted to the mononuclear cell population of a well-differentiated C2C12 culture, and that expression in the myotube-enriched population was virtually undetectable (Figure 5C). Consistent with our findings presented above, Id2 expression in this experiment was highest in myoblasts, and was significantly down-regulated after three days of differentiation, and undetectable in the presence of SB (20 μM) (Figure 5C).

Behaviour of Id and other proteins after fractionation of C2C12 cultures with and without SB

Markers of differentiation and proliferation were analysed in mononuclear cells (m) and multinucleate myotubes (t) following 3 days of differentiation in the absence or presence of SB. Mononuclear cells were enriched for markers of proliferation (PCNA [proliferating cell nuclear antigen] and cyclin D1), and the anti-differentiation proteins Id1 and Id2, reflecting the relatively uncommitted phenotype of this cell type. Myotubes were enriched for the early marker of muscle differentiation, p21.

In the SB-treated cultures, mononuclear cells were relatively deficient in Id proteins, especially Id1. Over-expression of Id proteins in low serum conditions inhibits C2C12 myogenic differentiation, and therefore we proposed that a majority of the mononuclear cells remaining in the SB-treated cultures would be in the early phases of differention. Consistent with this hypothesis was the increase in p21 in these cells. Changes in Id protein expression can be associated with, and necessary for cell proliferation. Mononuclear cells maintained their high levels of PCNA and cyclin D1, indicating that their proliferative status was unaffected by SB treatment. Therefore, the decrease in Id expression with SB treatment without changes in PCNA and cyclin D1 was consistent with a role for the Id proteins in regulating a switch between cell proliferation and differentiation, and not as a simple marker of this effect. This effect of SB treatment on Id protein levels within the mononuclear cells also contributes to the dramatic reduction in the level of Id proteins in unfractionated C2C12 lysates with SB treatment (Figure 6). This can now be explained by a combination of decreased Id expression in mononuclear cells and a reduction in the mononuclear/myotube cell ratio.

Analysis by microarray of transcriptional profiles in mononuclear and myotube cells from these cultures supported the hypothesis that SB treatment stimulated at least partial differentiation of the remaining mononuclear cells, i.e. genes that were normally myotube-specific were elevated in mononuclear cells from SB-treated cultures, and genes that were normally mononuclear cell-specific were repressed. The study indicates that Id proteins, particularly Id1, are maintained in the reserve cell population. Their known inhibitor activities on differentiation are likely to contribute to maintaining the undifferentiated state of reserve cells.

Effects of p38 in primary muscle cultures

We sought to address the same questions regarding the role of p38 in myogenesis but in a primary mouse muscle culture model.

Methodology

Single myofibres from the EDL (extensor digitorum longus) and soleus muscles of the *Myf5* $^{nlacZ/+}$ mouse were prepared as previously described (Rosenblatt, J.D., A.I. Lunt, D.J. Parry, and T.A. Partridge. 1995. Culturing satellite cells from living single muscle fiber explants. *In Vitro Cell Dev Biol Anim* 31: 773-9). The single fibres were cleaned and plated on a matrigel coated cell culture dish. After 24 hours the cells began to migrate from the fibres and started to divide. To increase proliferation of satellite cells, the medium used consisted of 20% of FBS supplemented with 0.05% chicken embryo extract and bFGF (10 nM). After four days in culture, cells were trypsinised and plated on 24 well plates coated with matrigel. Half of the cells were maintained in a proliferation medium containing 20% FBS (no bFGF) and the other half of the cells were switched to a differentiation medium containing 2% horse serum. Each set of cells was grown in the absence or presence of SB (20 μM) which was added at the onset of differentiation and replenished daily thereafter.

Results

High density cultures

Cells were plated at high density in 24-well dishes then differentiated ± SB as outlined above. After 24 hours, the changes in the SB-treated cultures were striking. Control cells began to differentiate but there were numerous single rounded cells sitting on top of differentiated cells (Figure 7A). In the SB-treated cultures, myoblasts started to elongate and apparently differentiated in higher numbers (Figure 7B). In addition, there were far fewer single rounded cells (at least four times less) compared with control cultures. Over the following three days, these observations became more pronounced (Figures 7C-F). There was greater differentiation in the SB-treated cells with respect to alignment of the elongated cells, although there appeared to be an inhibition to complete fusion. Despite the apparent promotion of differentiation, there were still a few mononuclear cells present after four days of differentiation in the SB-treated culture, although far fewer than in the control cultures.

CONCLUSIONS

These data in SB-treated primary mouse myoblasts are very similar to the phenotypic observations described in our C2C12 cultures. Inhibition of p38 with SB rapidly induced a marked phenotypic alteration with apparent increases in the proportion of differentiating myoblasts but also decreases in the number of mononuclear cells. Furthermore, a small subpopulation of mononuclear cells remained apparently undifferentiated in the presence of SB. All of the observations in primary mouse muscle cultures are consistent with our data and our hypothesis that p38 plays a dual role in myogenesis, and in particular, a novel role in the establishment of a stem-like cell population in myogenic cultures.

SUMMARY

Model of p38 and myogenesis

We have demonstrated that the p38 MAPK pathway is important in skeletal myogenesis *in vitro*: p38 appears to be necessary for the creation and/or maintenance of the reserve cell population, and required for terminal fusion of differentiated myocytes. Indeed, we note that the C2C12 model of skeletal myogenesis represents a valid template for studying the process of self-renewal in at least one class of muscle stem cells.

In theory, a myoblast could undergo symmetric and/or asymmetric cell divisions. If a symmetric division occurs, the common progeny could either be MyoD-positive myoblasts, or MyoD-negative reserve cells. Alternatively, myoblasts may divide asymmetrically, giving rise to one reserve cell and one cell that is more committed. We have examined these possible types of cell division during the two main phases of myogenesis: proliferation and differentiation. Our model recognises that conditions of high or low serum will increase or decrease the probability of a particular form of division occurring, rather than inducing an all-or-none phenomenon.

This invention relates to how skeletal muscles and other tissues renew their stem cell populations in normal physiology and disease. The benefit of this work lies in its potential value to cell-based or drug therapies for degenerative diseases of the aged, such as stroke, ischaemic heart disease, and skeletal atrophy, as well as myopathies, burns, blood disorders and any other condition in which tissue regeneration via stem cells occurs.

The invention sheds light on the molecular basis of stem cell self-renewal in a muscle satellite cell culture system (C2C12), and in particular the role of the mitogen-activated protein kinase (MAPK) p38 and Notch in this process. We have found that p38 and Notch are important components of the regulatory machinery that sets the proportion of self-renewing (reserve) and differentiating cells in this system. This may occur as a dynamic equilibrium, capable of modulation by physiological inputs. We also propose that an understanding of self-renewal in this model will have relevance to other stem cell systems. Thus, important aspects are effects of modifying expression of p38 and Notch at the early stages of C2C12 differentiation; links between C2C12 reserve cells and *in vivo* muscle stem cells; and links between reserve cells and other stem cell systems.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Claims:

1. A method for controlling precursor cell differentiation comprising treating a precursor cell with an agent that affects activity of mitogen-activated protein kinase (MAPK) p38 or signalling of Notch in the cell such that the cell undergoes differentiation or undergoes self renewal.
2. The method according to claim 1 wherein the precursor cell is a stem cell or a myogenic cell line.
3. The method according to claim 3 wherein the myogenic cell line is C2C12.
4. The method according to any one of claims 1 to 3 wherein the MAPK p38 is p38α or p38β.
5. The method according to any one of claims 1 to 4 wherein the agent that affects activity of MAPK p38 is SB203580 (SB).
6. The method according to any one of claims 1 to 4 wherein the agent is an inhibitor of MAPK p38 or an activator of MAPK p38.
7. The method according to claim 6 wherein the inhibitor is selected from the group consisting of SKF 86002, SKF 86002 derivatives, HEP689, VX-745, and mixtures thereof.
8. The method according to any one of claims 1 to 3 wherein the agent that affects Notch signalling in the cell is DFK-167, MG132 or mixtures thereof.
9. A cell model for studying cell differentiation and self renewal comprising a cell modified to alter its sensitivity to an agent that affects activity of MAPK p38 or Notch signalling in the cell.
10. The cell model according to claim 9 wherein the MAPK p38 is p38α or p38β.
11. The cell model according to claim 9 or 10 selected from the group consisting of a modified a precursor cell, a modified stem cell, and a modified C2C12 cell.
12. The cell model according to any one of claims 9 to 11 wherein the agent is selected from the group consisting of SB203580, SKF 86002, SKF 86002 derivatives, HEP689, VX-745, DFK-167, MG132, gamma secretase inhibitor X, MDL28170, and mixtures thereof.
13. The cell model according to claim 12 wherein the agent is SB203580.

14. The cell model according to any one of claims 9 to 13 being modified to alter expression of MAPK p38 or Notch signalling during pre-differentiation of the cell at which time reserve cells are formed, or during post-differentiation of the cell.

15. The cell model according to claim 14 being modified to over express, constitutively express, inducibly express, or substantially not express MAPK p38α, p38β or Notch.

16. A method for assaying an agent for a capability of influencing cell differentiation or self renewal, the method comprising:

providing a cell model according to any one of claims 9 to 15;

treating the cell model with an agent; and

determining the affect of the agent on the differentiation or self renewal of the cell model.

Figure 1:
(A) Model for role of p38 during myogenesis.

(B) Model for regulation of reserve cells

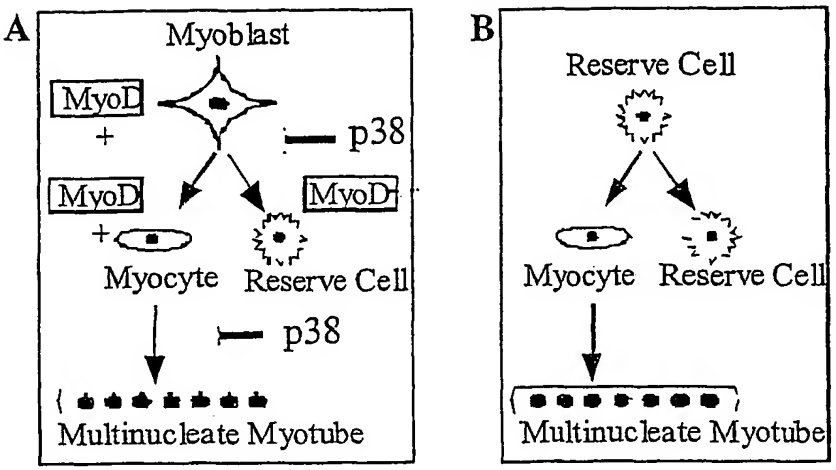
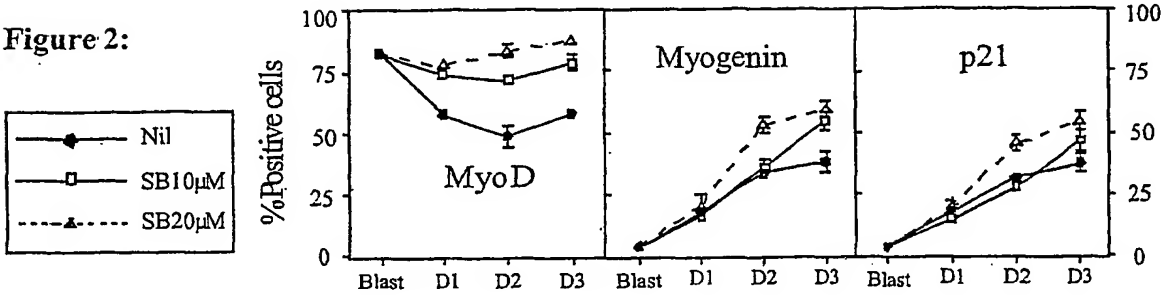


Figure 2:



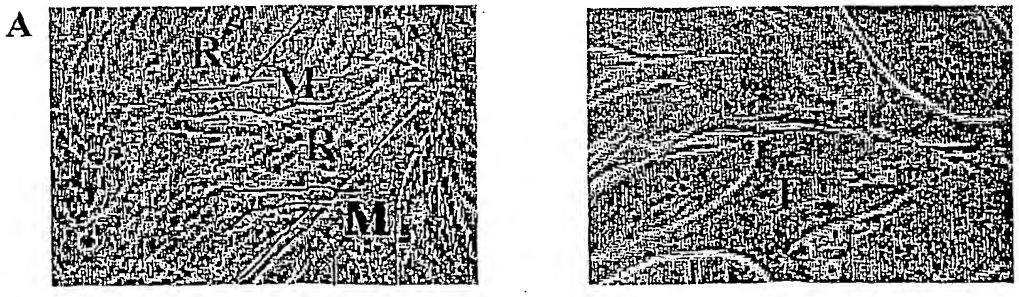


Figure 3: MLC p38 DR clone: (A) Differentiation at day 4 shows a complex mix of multinucleate myotubes (M - some outlined) and reserve cells (R) and; (B) differentiation at day4 in the presence of SB 20mM shows virtual "clearing" of reserve cells (*) with fusion into massive myosacs (F).

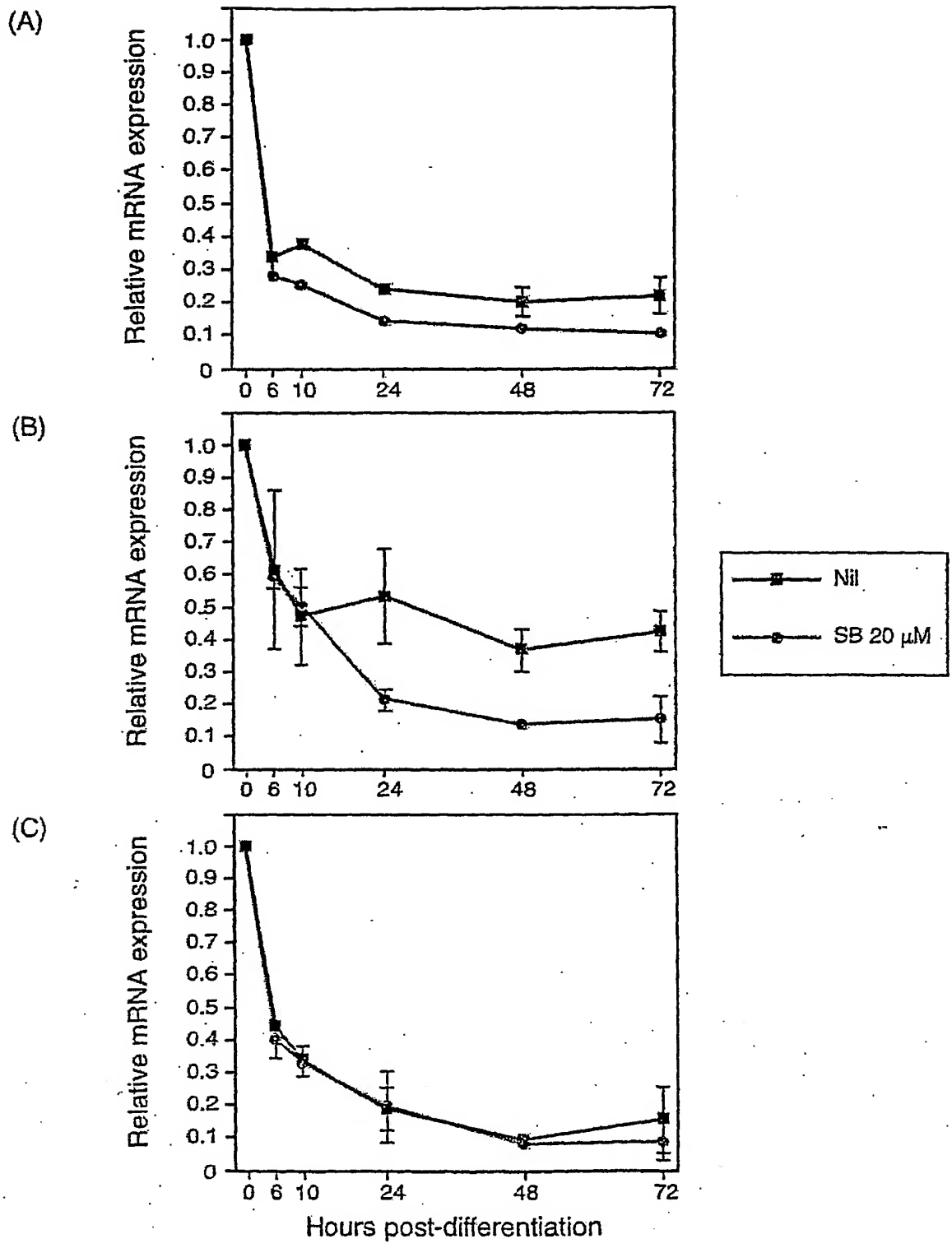


Figure 4: *Id* (*1-3*) mRNA expression is down-regulated during differentiation.

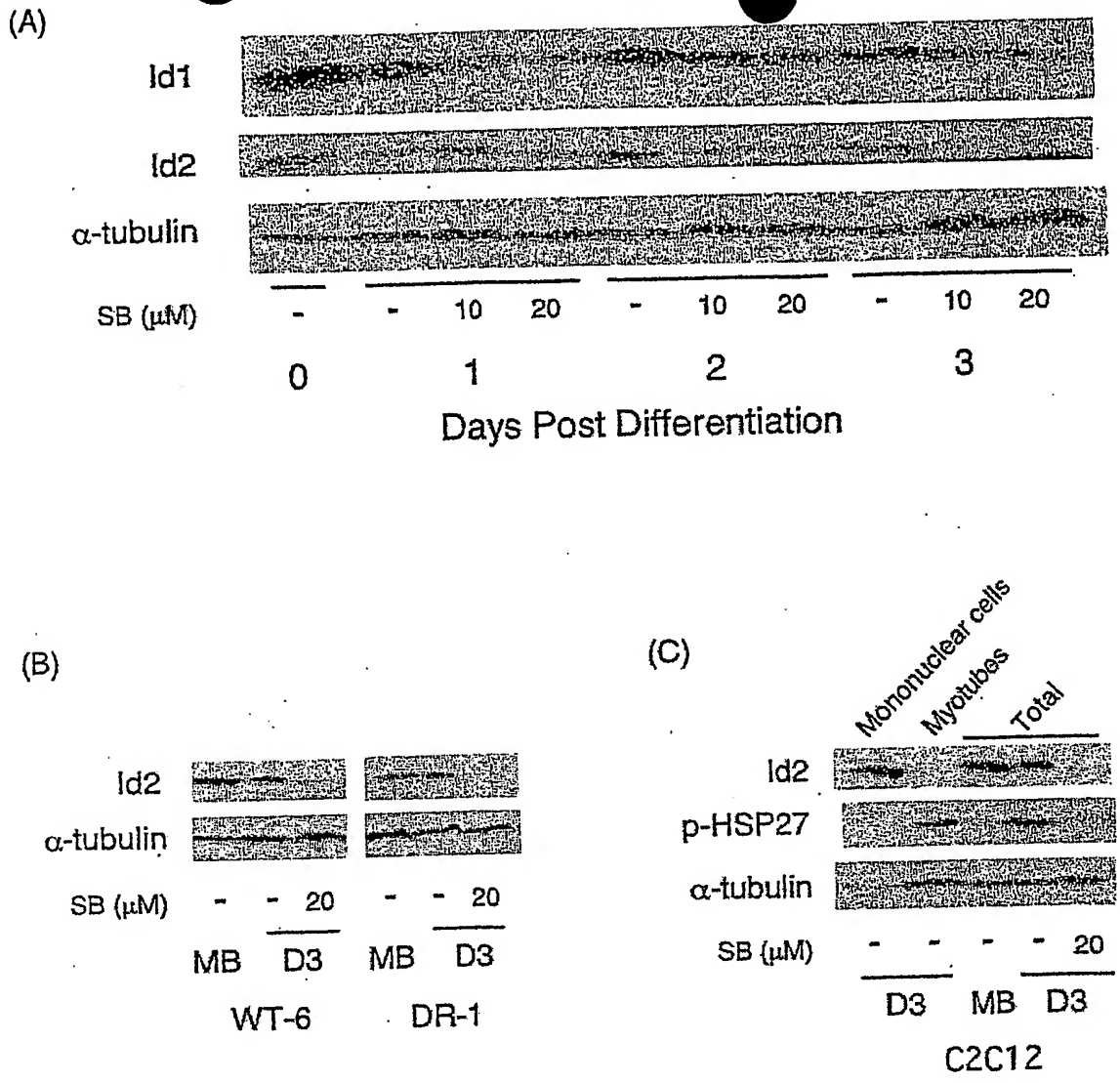


Figure 5: Western blot analyses of Id1 and Id2 protein expression.

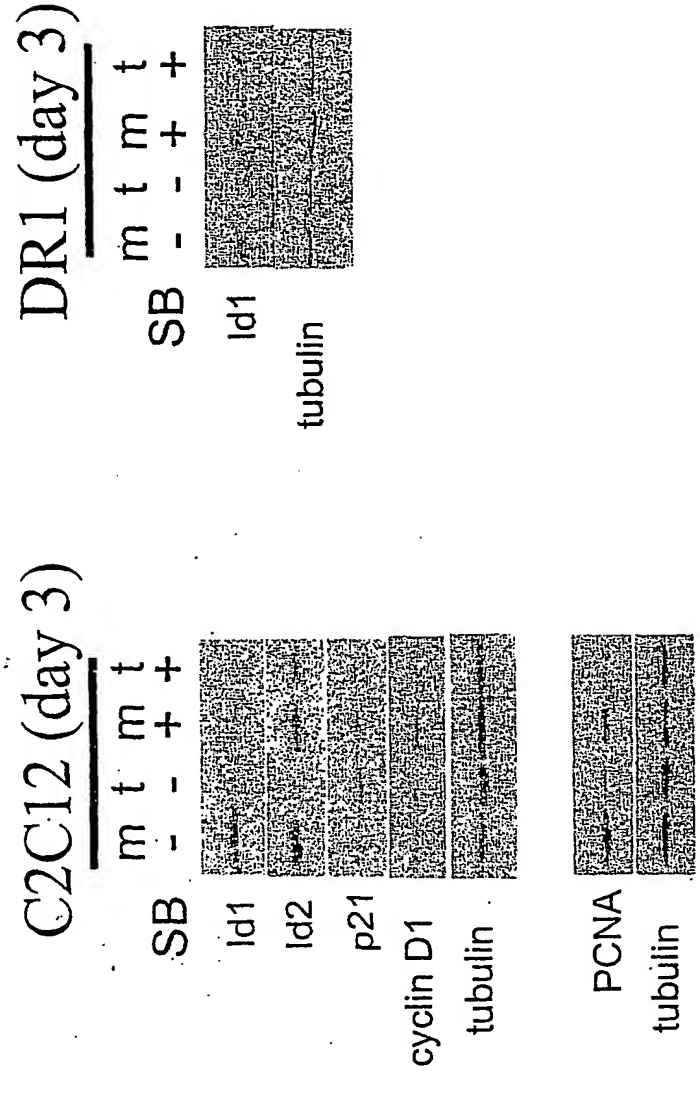

C2C12 (day 3)
m t m t
SB - - + +
Id1
Id2
p21
cyclin D1
tubulin
PCNA
tubulin
DR1 (day 3)
m t m t
SB - - + +
Id1
tubulin

Figure 6

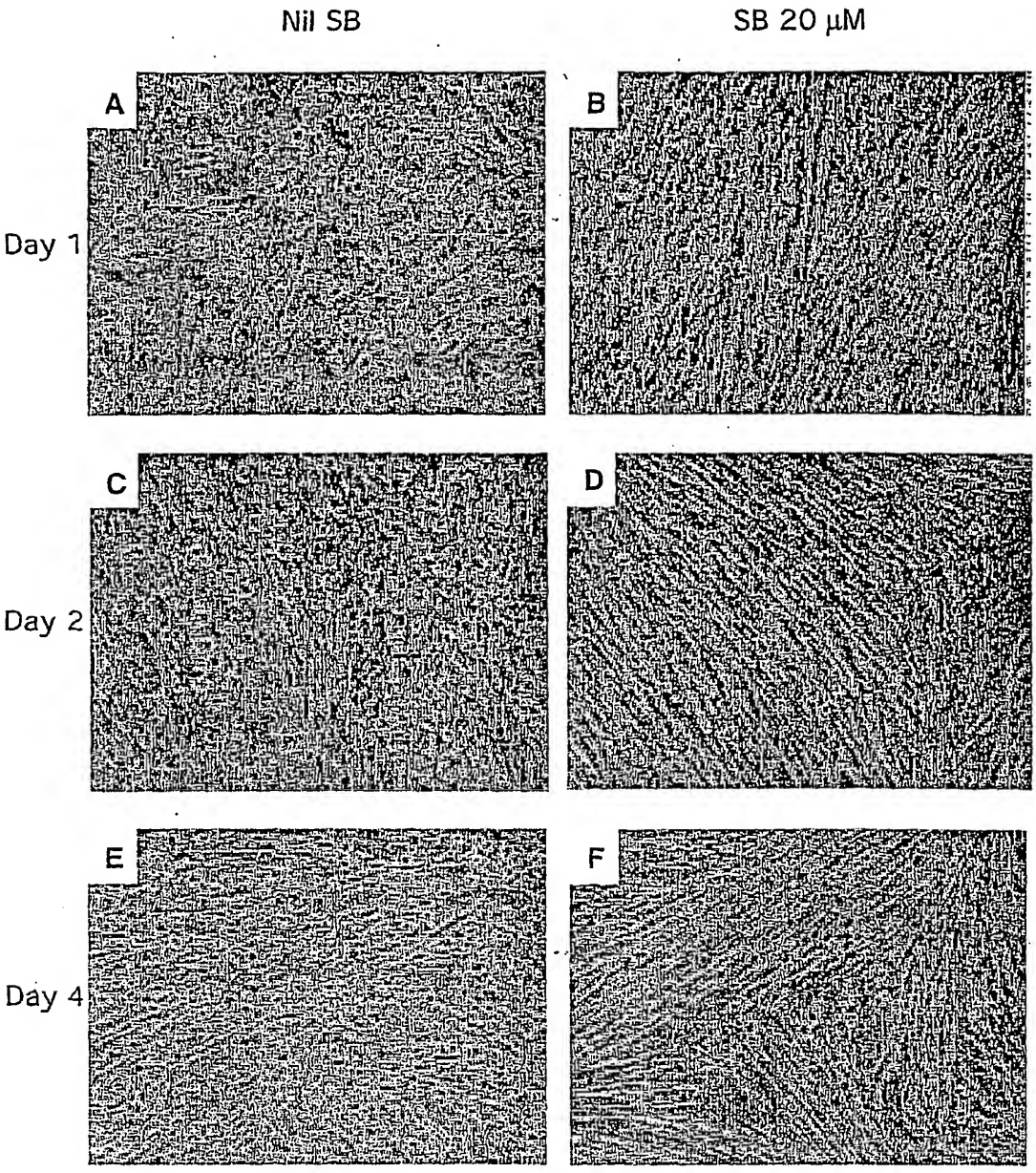


Figure 7: Inhibition of p38 with SB increases the number of thin and elongated myocytes and induces a loss of mononuclear cells in high density cultures of primary mouse myoblasts.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU03/00518

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C12N 005/02; 005/06; 005/08; 005/10; 015/63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
SEE ELECTRONIC DATABASE BOX BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SEE ELECTRONIC DATABASE BOX BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPIDS CA MEDLINE BIOSIS: STEM CELLS, CELL DIFFERENTIATION, PROLIFERATION, PROPAGATION, RENEWAL, PROGENITOR, PRECURSOR, NOTCH, P38, SATELLITE, MYOGENIC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Zetser A et al (1999). p38 mitogen-activated protein kinase pathway promotoes skeletal muscle differentiation: participation of the MEF2C transcription factor. The Journal of Biological Chemistry 274(8): 5193–5200. See entire document	1–7, 9–16
X	Kuroda K et al (1999). Delta-induced Notch signalling mediated by RBP-J inhibits MyoD expression and myogenesis. The Journal of Biological Chemistry 274(11): 7238-7244. See entire document.	1–3, 8, 9, 11, 14–16
P X	WO 02/094842 A2 (UNIVERSITY OF WESTERN ONTARIO) 28 November 2002. See entire document.	1–7, 9–16

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 May 2003	12 JUN 2003
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer PHILIPPA WYRDEMAN Telephone No : (02) 6283 2554

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU03/00518

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P X	WO 02/059285 A1 (FRED HUTCHINSON CANCER RESEARCH CENTER) 1 August 2002. See entire document.	1, 2, 8, 9, 11, 14–16
P X	WO 02/077204 A2 (AXORDIA LIMITED) 3 October 2002. See entire document.	1–3, 8, 9, 11, 14–16
P X	Conboy IM and Rando TA (Sept 2002). The regulation of Notch signalling controls satellite cell activation and cell fate determination in postnatal myogenesis. Developmental Cell 3(3): 397–409. See entire document.	1–3, 8, 9, 11, 14–16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU03/00518

Box I **Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :

because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos :

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos :

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II **Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU03/00518

Supplemental Box
(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No: II

The international application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept. In coming to this conclusion the International Searching Authority has found that there are different inventions as follows:

1. Claims 1–3, 9, 11, 12, 14–16 (all partially); claims 4–7, 10, 13 (completely). It is considered that modulation of p38 activity to control cell differentiation or self renewal comprises a first "special technical feature".

2. Claims 1–3, 9, 11, 12, 14–16 (all partially); claim 8 (completely). It is considered that modulation of Notch signalling to control cell differentiation or self renewal comprises a second "special technical feature".

Since the abovementioned groups of claims do not share any of the technical features identified, a "technical relationship" between the inventions, as defined in PCT rule 13.2 does not exist. Accordingly the international application does not relate to one invention or to a single inventive concept, a priori.

However since all these inventions share the same classification under the IPC they could be searched together without effort which would warrant an additional fee. Therefore all the inventions have been searched without extra charge.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU03/00518

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member
WO	02/094842 A2	NONE
WO	02/059285 A1	NONE
WO	02/077204 A2	NONE